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Article

A Role for the IκB Family Member Bcl-3 in the Control of Central Immunologic Tolerance

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SUMMARY

Bcl-3 is a member of the family of IκB inhibitors. Unlike the classical, cytoplasmic IκBs, Bcl-3 does not inhibit RelA- or c-Rel-containing NF-κB transcription factor dimers. Instead, Bcl-3 can enter the nucleus and modulate NF-κB activity, although the underlying mechanism and physiologic function remain largely unknown. Here we identified Bcl-3 as a regulator of immunologic tolerance to self. In parallel with NF-κB2, Bcl-3 functions within stroma to generate medullary thymic epithelial cells, which are essential for negative selection of autoreactive T cells. Loss of both NF-κB2 and Bcl-3, but not either one alone, led to a profound breakdown in central tolerance resulting in rapid and fatal multiorgan inflammation. These data reveal extensive utilization of the NF-κB system to promote central tolerance in the thymus, in apparent contrast with the well-known roles of NF-κB to promote inflammation and autoimmunity in the periphery.

INTRODUCTION

The NF-κB transcription factor family is central to host defense against pathogenic insults. NF-κB functions as a primary intracellular mediator of numerous innate, inflammatory, and adaptive immune signals (Hayden and Ghosh, 2004). NF-κB has been recognized as well for its essential roles during development and maintenance of the immune system, prior to and apparently independent of encounter with pathogens (Claudio et al., 2006; Siebenlist et al., 2005).

NF-κB factors are also required for proper development and function of secondary lymphoid organs. Mice deficient in RelB or NF-κB2 or which have inactivating mutations in NIK (*aly/aly* mice) or in IκB kinase α (IKKα, also known as IKK1) display an overlapping spectrum of defects in structure and function of secondary lymphoid organs (Franzoso et al., 1998; Matsushima et al., 2001; Paxian et al., 2002; Senftleben et al., 2001; Weih and Caamano, 2003). These mutant mice fail to form proper B cell follicles and differentiated follicular dendritic cell networks (FDCs), and, upon challenge, they fail to form proper germinal centers in spleens. They also lack Peyer's patches and, depending on the deficiency of the particular NF-κB factor, they may lack some or all lymph nodes (see below). These deficiencies are due in large part to impaired stromal cell functions. RelB, NF-κB2, NIK, and IKKα are all components of the nonclassical pathway for NF-κB activation. It then follows that the nonclassical pathway in stromal cells must be essential for proper lymphoid organogenesis. Although the nonclassical pathway is disrupted in NF-κB2-deficient mice, loss of this factor results in a substantially milder phenotype. For example, lymph node structures are present (Franzoso et al., 1998; Weih and Caamano, 2003).

Lymphotoxin β receptors (LTβR) are expressed primarily on stromal cells and upon stimulation engage the nonclassical pathway for NF-κB activation (Basak et al., 2007; Muller and Siebenlist, 2003). Consistent with a role for LTβR-mediated activation of the nonclassical pathway in stromal cells during lymphoid organogenesis, mutant mouse models deficient in LTβR or its main ligand, $(LT\alpha)_1(LT\beta)_2$, have defects similar to those described above for mice lacking components of the nonclassical pathway (Hehlgans and Pfeffer, 2005; Matsushima et al., 2001).

Mice blocked in signaling via the LTβR or via the nonclassical pathway share defects in addition to those associated with lymphoid organogenesis. Mice deficient in RelB, NIK (*aly/aly*), and, possibly to a lesser degree, LTβR signaling display thymic defects and develop marked multiorgan lymphocytic infiltrations early in life (Boehm et al., 2003; Chin et al., 2003; Hehlgans and Pfeffer, 2005; Kajiura et al., 2004; Kinoshita et al., 2006; Weih et al., 1995). In the case of RelB-deficient mice, the inflammatory condition can be fatal as early as 7–8 weeks of age, although there is wide range and mice may survive for many more months. Such pathology is not seen in NF-κB2-deficient mice, which develop only mild organ infiltrations relatively late in life, without any effect on their life span (Franzoso et al., 1998; Weih and Caamano, 2003; Zhang et al., 2006; Zhu et al., 2006). It is likely that NF-κB2-deficient mice are largely protected from severe pathology because they retain RelB activity. The absence of the RelB inhibitor p100 allows p50-RelB dimers to enter nuclei where they may partially compensate for loss of RelB dimers (including p52-RelB) activated via the nonclassical pathway (Claudio et al., 2002; Muller and Siebenlist, 2003).

In addition to the mutant mouse models discussed here, Bcl-3-deficient mice display defects in secondary lymphoid organs (Franzoso et al., 1997a; Paxian et al., 2002). These defects are partly similar to but even milder than those observed in NF-κB2-deficient mice. Bcl-3 is structurally related to the IκBα, β, and ε inhibitors but is distinct in several critical ways. Bcl-3 is often found in the nucleus, contains domains that can stimulate transcription, interacts predominantly with p50 and p52 homodimers, and has been reported to directly or indirectly transactivate or repress gene expression via κB elements (Bours et al., 1993; Franzoso et al., 1992, 1993; Fujita et al., 1993; Hayden and Ghosh, 2004). Bcl-3 has also been implicated in various gene regulatory models and biologic scenarios (Corn et al., 2005; Kashatus et al., 2006; Massoumi et al., 2006; Mitchell et al., 2002; Viatour et al., 2005; Wessells et al., 2004), but its actual physiologic mechanism(s) of action and in vivo targets remain essentially unknown. Because Bcl-3 is not known to have a direct role in the nonclassical NF-κB signal activation pathway, the available evidence suggests two basic models whereby Bcl-3 might influence secondary lymphoid organogenesis: Bcl-3 could modulate NF-κB activity via direct interaction with p52-NF-κB2, or Bcl-3 could function in a pathway separate from but partially redundant with NF-κB2. To address these possibilities, we generated mice deficient in both proteins. In addition to a complete block in secondary lymphoid organogenesis, the doubly deficient mice developed severe lymphocytic infiltrates in multiple organs. Therefore, Bcl-3 and NF-κB2 have redundant biologic effects that are revealed only in the absence of both proteins. We further demonstrated a profound impairment of central T cell tolerance in these mutant mice, which could be traced to defects in thymic stromal cells.

RESULTS

Phenotype of Mice with NF-κB2 and Bcl-3 Deficiency

We generated NF-κB2, Bcl-3 doubly deficient (*Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$) mice to investigate whether Bcl-3 interacts with NF-κB2 to modulate its activity or whether these proteins function in distinct pathways that may nevertheless converge on some common targets. Mice deficient in either one of these proteins had fully normal life spans and presented with limited phenotypic defects, mostly resulting from mildly impaired secondary lymphoid organogenesis, although *Nfkb2*$^{-/-}$ but not *Bcl3*$^{-/-}$ mice developed mild inflammation in some organs later in life. Unexpectedly, loss of both NF-κB2 and Bcl-3 proved fatal by 4 weeks of age (C57BL/6 background; 3–7 weeks on mixed background). These *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were also small in size, which became apparent within 2 weeks of birth. By the time of weaning, the mutant mice began to develop ruffled fur, scaly skin, a hunched posture, and squinting eyes, among other symptoms. Further analysis revealed an absence of all lymph nodes, including mesenteric lymph nodes, and gradual loss of the thymus, beginning by about 2 weeks after birth (Figure S1 in the Supplemental Data available online). Singly deficient mutants had a normal-sized thymus and contained lymph nodes. Although most lymph nodes were reduced in cellularity in *Nfkb2*$^{-/-}$ mice, mesenteric lymph nodes appeared completely normal in both singly deficient mice (Franzoso et al., 1997a, 1998; Paxian et al., 2002; Weih and Caamano, 2003). The loss of NF-κB2 and Bcl-3 therefore generated a new phenotype, supporting the notion that Bcl-3 and NF-κB2 must have some redundant functional outcomes, while working independently of each other.

The rapid death of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice was surprising and implied activities of the proteins encoded by these genes in addition to those involved in secondary lymphoid organogenesis. The appearance of the mice suggested an inflammatory condition, which was confirmed by tissue analyses. Examination of paraffin-embedded, hematoxylin and eosin (H&E)-stained tissue sections from liver, lung, and skin revealed inflammatory infiltrates (Figure 1A), which contained many T cells (not shown). We therefore examined the state of splenic T cells in 3- to 4-week-old *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. Splenocytes from these mice contained relatively more T cells because mature B cell numbers were severely reduced (Figure 1B; the majority of CD4^{-}CD8^{-} cells are B cells). Among the T cells, there was a dramatic increase in the relative amounts of CD44^{+}CD62L^{-} effector and memory cells in both the CD4 and CD8 compartments when compared to *Nfkb2*$^{-/-}$ control mice (*Bcl3*$^{-/-}$ mice were similar to the *Nfkb2*$^{-/-}$ controls; not shown). The *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ T cell population also contained more activated cells as judged by increased expression of the activation markers CD69 and CD25 (not shown). The increase in effector and memory (and activated) T cells attested to an ongoing immune reaction in these mice.

Pathology of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice Is Mediated by T Cells

The presence of lymphocytic infiltrates in multiple organs and the pronounced increase in effector and memory T cells suggested autoimmune reactivity. To test whether T cells from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mutants could react with self-antigens, CD4^{+} T cells isolated by negative selection were incubated with wild-type antigen-presenting cells (APCs) isolated from syngeneic mice (Figure 2A). T-depleted, irradiated splenocytes served as APCs in this "syngeneic" mixed lymphocyte reaction (MLR). In the absence of APCs, the T cells did not proliferate, as judged by thymidine incorporation, except for a very low amount of proliferation in T cells from the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. In the presence of (self-antigen-presenting) syngeneic APCs, T cells isolated from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice proliferated at a substantially higher rate than did T cells from the two single-deficient mice. *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ T cells did not intrinsically proliferate better because nonspecific stimulation with CD3 antibodies did not result in their preferential growth (data not shown). Together, these data provided further evidence for the presence of substantial numbers of autoreactive T cells in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice.

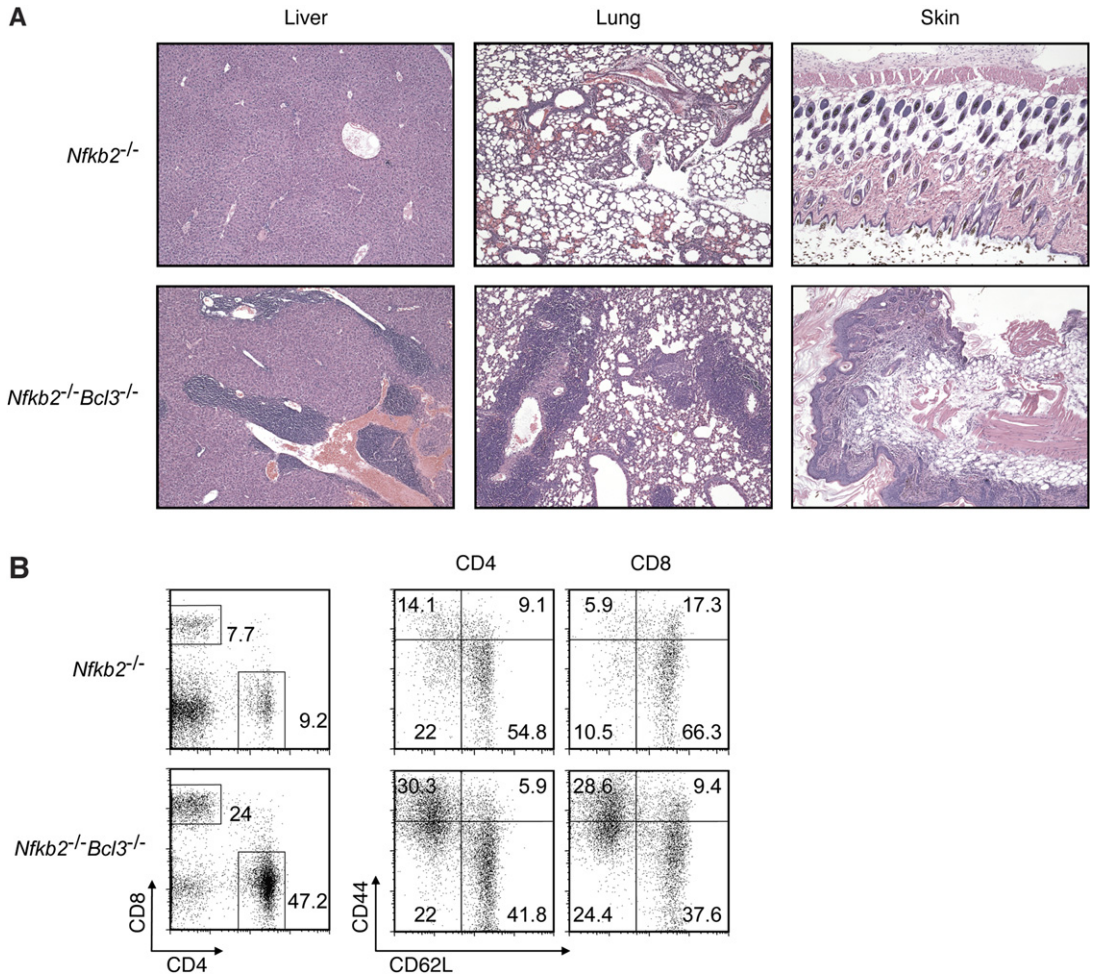

Figure 1. *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice Develop Severe Multiorgan Inflammation

(A) Tissue sections from liver, lung, and skin of nearly 4-week-old *Nfkb2*$^{-/-}$ and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were stained with hematoxylin and eosin (H&E).

(B) Increased numbers of effector and memory and autoreactive T cells in spleens of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. Splenocytes from 26-day-old *Nfkb2*$^{-/-}$ and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were examined with flow cytometry for the expression of CD4 and CD8 (left) and for expression of CD44 and CD62L on CD4^{+} and CD8^{+} T cells (right).

Data shown are representative of at least five independent experiments.

To demonstrate more directly that autoreactive T cells were responsible for the pathology of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, we isolated CD4^{+} T cells from these mutant mice and injected them intravenously into sublethally irradiated *Rag1*$^{-/-}$ mice, which bear no T or B cells of their own. The recipients showed no obvious ill effects after transfer of CD4^{+} T cells from *Nfkb2*$^{-/-}$ or *Bcl3*$^{-/-}$ mice, but all mice receiving CD4^{+} T cells from the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice succumbed to the inflammatory condition 8–12 weeks after transfer (Figure 2B). We observed lymphocytic infiltrates in these mice, especially in lung and liver, although the inflammation appeared less than in the original *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mutant mice (data not shown).

To confirm the requirement of (auto-) antigen-specific T cells in the development of multiorgan inflammation, we generated *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice bearing a transgene that directs the expression of a T cell receptor (TCR) that is MHC class I restricted and specific for a peptide from the chicken ovalbumin protein (OT-I transgene) (Clarke et al., 2000). Allelic exclusion in OT-I transgenic animals severely restricts the appearance of endogenous TCRs, and therefore the presence of this transgene in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice should largely block the appearance of autoreactive T cells and thus prevent the development of pathology. As predicted, the OT-I transgene "rescued" the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice and these mice showed no serious pathology even by 12 weeks of age, with only sporadic and limited infiltrates in lung and liver at that time (Figure 2C; we never observed skin infiltrates). The OT-I transgene also prevented the dramatic rise in effector and memory T cells (most T cells on this background are CD8; data not shown). Together, these data support the hypothesis that autoantigen-specific T cells mediated the pathology of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice.

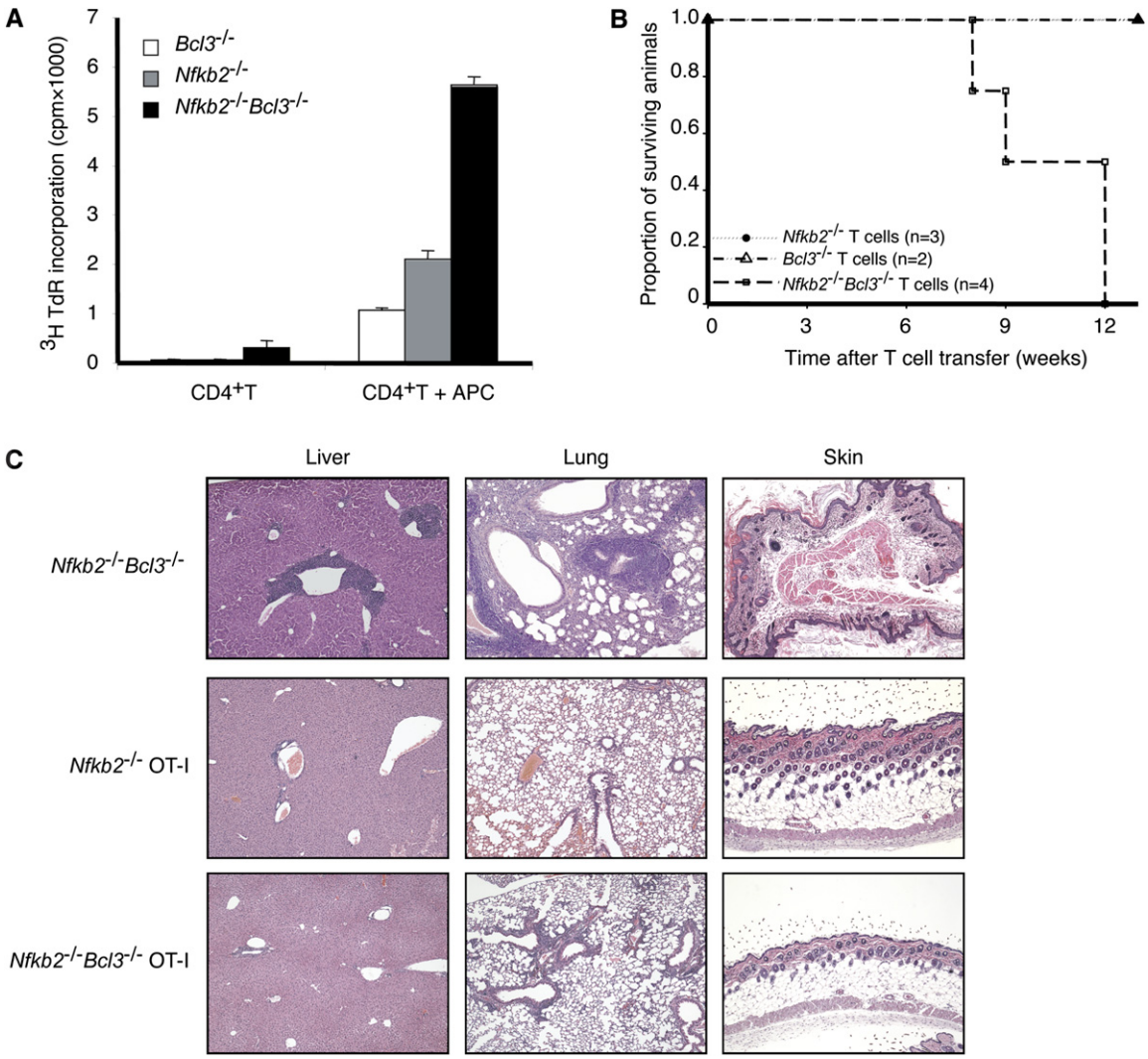

Figure 2. T Cells Mediate Fatal Multiorgan Inflammation in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice

(A) CD4$^+$ T cells purified from spleens of 26-day-old *Bcl3*$^{-/-}$, *Nfkb2*$^{-/-}$, and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were cultured with or without irradiated syngeneic antigen-presenting cells (APCs) (see Experimental Procedures) and pulsed with [^{3}H]thymidine. Data are the mean of triplicates ± SD and similar results were obtained in three independent experiments.

(B) CD4$^+$ T cells were purified from nearly 4-week-old *Bcl3*$^{-/-}$, *Nfkb2*$^{-/-}$, and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice and transferred into sublethally irradiated *Rag1*$^{-/-}$ mice (5 × 10^6 T cells per mouse). Recipient mice were monitored daily and the surviving proportion was plotted. Number of independent experiments is indicated (n) ($p = 0.03$ for transfer of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ T cells when compared to transfer of *Bcl3*$^{-/-}$ and *Nfkb2*$^{-/-}$ T cells).

(C) The T cell receptor OT-I transgene prevents fatal multiorgan inflammation in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. Liver, lung, and skin sections from nearly 4-week-old *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ and 12-week-old *Nfkb2*$^{-/-}$ OT-I and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I mice were stained with H&E. Data are representative of at least four independent experiments.

T Regulatory Cells

T regulatory (Treg) cells are critical for maintenance of peripheral tolerance to self-antigens (Ziegler, 2006). Because the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were unable to control autoimmune reactions, we postulated that Treg cells might be fewer or functionally impaired in these mice. Flow cytometric analysis of splenic T cells from 26-day-old *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ animals did not show reduced numbers of CD4$^+$CD25$^+$ Foxp3$^+$ Treg cells (Figure 3A, right). We also analyzed the thymus of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice at 11 days of age (well before the thymus shrinks) for the presence of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, and we noted reduced numbers of these cells at this early time relative to *Nfkb2*$^{-/-}$ mice (Figure 3A, left). Because peripheral Treg cell numbers just prior to death were not reduced, the relevance of reduced numbers of Treg cells in the thymus shortly after birth is unclear, but may well be important, especially because the thymus started to shrink at 2 weeks of age (see Discussion).

To test the inhibitory activity of peripheral Treg cells, wild-type CD4$^+$CD25$^-$ naive splenic T cells were activated in the presence of varied amounts of CD4$^+$CD25$^+$ splenic

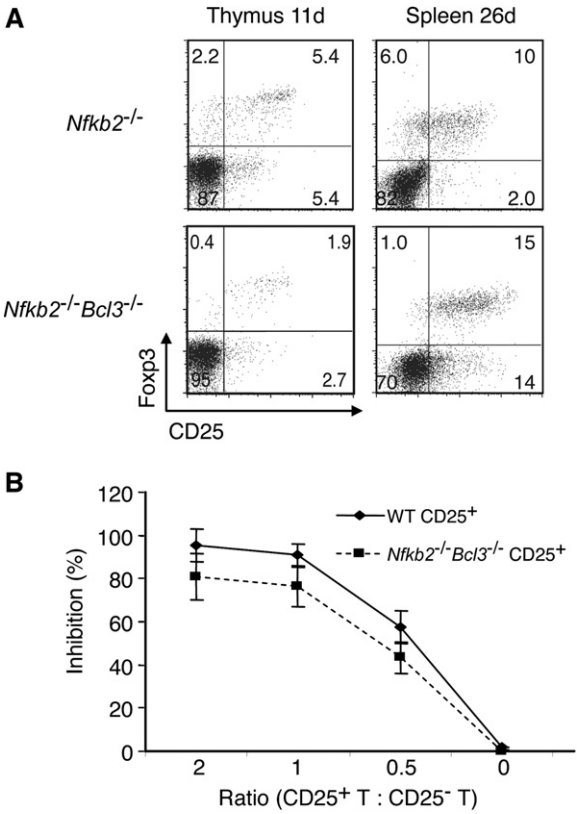

Figure 3. Analysis of T Regulatory Cells

(A) 11-day-old *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice have reduced numbers of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in thymus, whereas 26-day-old *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice have above normal numbers of Treg cells in spleen. Thymocytes and splenocytes were gated on CD4$^+$CD8$^-$ and CD4$^+$, respectively, and analyzed for expression of Foxp3 and CD25 with flow cytometry. Example shown is representative of five independent experiments.

(B) CD4$^+$CD25$^+$ splenic Treg cells of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice have near-normal suppressive activity in vitro. Wild-type CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3 and pulsed with [^{3}H]thymidine in the presence of syngeneic antigen-presenting cells (APCs) as well as freshly isolated CD4$^+$CD25$^+$ Treg cells from spleens of wild-type or *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice (see Experimental Procedures). 2-fold dilutions of suppressive Treg cells were used as shown. Data are the mean of triplicates ± SD and are representative of three independent experiments.

Treg cells from either wild-type or *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. The CD25$^+$ Treg cells isolated from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ animals did not appear to be markedly impaired in their ability to suppress anti-CD3-induced proliferation of wild-type CD25$^-$ T cells (Figure 3B). Together, these data suggest that the generation and function of peripheral Treg cells is not globally defective in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, although thymus-derived Treg cells may be partially impaired.

Primary Defect of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice in Stromal Cells

As demonstrated above, autoreactive T cells from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice could induce serious pathology upon transfer. It remained to be determined, however, whether the causative defect resided in T cells, other hematopoietic cells, stromal cells, or a combination of cell types, given that two proteins were involved. To address this question, we performed reciprocal bone-marrow transplants. First, we adoptively transferred bone marrow from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice into lethally irradiated *Rag1*$^{-/-}$ (Figure 4A). In initial experiments, 3 of 14 recipients of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ bone marrow developed serious pathology by 12 weeks after transfer, whereas the remaining recipients showed no marked problems even at 14 weeks. However, all of the mice receiving *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ bone marrow experienced a transient weight loss after transfer. In control experiments, none of the *Rag1*$^{-/-}$ mice receiving bone marrow from wild-type, *Nfkb2*$^{-/-}$, or *Bcl3*$^{-/-}$ mice developed pathology or experienced weight loss. Because whole bone marrow was transferred in these experiments, this included some already fully matured T cells. Sufficient numbers of such mature T cells might have been transferred to cause overt problems in 3 out of 14 recipients and at least transient weight loss in all recipients. To more stringently test whether hematopoietic stem cells (as opposed to already mature T cells) from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice could cause pathology, we first depleted bone marrow of CD4$^+$ and CD8$^+$ T cells before transfer into lethally irradiated mice, in this case *Nfkb2*$^{-/-}$ mice. None of the recipient mice developed substantial pathology or experienced weight loss by 12 weeks after transfer and even later (a few mice were observed for several additional weeks) (Figure 4A). Further analysis of mice receiving T-depleted bone marrow showed no inflammatory infiltrates or increases in effector and memory cells (bottom panels of Figure 4B and data not shown). Together, these findings led us to conclude that hematopoietic stem cells from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were not sufficient to cause the inflammatory condition. However, it remained possible that defects intrinsic to hematopoietic cells were nevertheless necessary to develop such pathology.

To determine whether stromal cells from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were necessary and sufficient to cause multiorgan infiltrates, we performed the reciprocal experiment, transferring wild-type bone marrow into *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. Although the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice could not serve as recipients because their health deteriorated rapidly after birth, the OT-I transgenic *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were ideal for this purpose because the TCR transgene largely prevented development of an inflammatory condition in these mice (see above). Wild-type bone marrow was transferred into lethally irradiated, *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I mice, generating chimeric mice in which hematopoietic cells were genetically wild-type (and without transgene), whereas stromal cells were of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ origin. All recipient mice succumbed between 5 and 10 weeks after transfer (Figure 4C). In control transfers of bone marrow from wild-type mice into OT-I transgenic, *Nfkb2*$^{-/-}$ mice, no overt pathology was evident in any recipients. Tissue analysis of OT-I *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice that had received wild-type bone marrow confirmed severe inflammatory infiltrates in lung, liver, and skin

(Figure 4B, top row). OT-I, *Nfkb2*$^{-/-}$ recipients of wild-type bone marrow (controls) did not develop this pathology, although mild infiltration of lung and liver could be observed in a few individuals (Figure 4B, middle row). Consistent with these phenotypes, *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice with wild-type hematopoietic cells had dramatically increased CD44^{+}CD62L^{-} effector and memory T cell populations compared with control *Nfkb2*$^{-/-}$ mice with wild-type hematopoietic cells (Figure 4D). The latter control chimeric mice variably exhibited somewhat elevated numbers of effector and memory cells when compared to normal mice, consistent with variably observed mild inflammation in these chimeric mice, although overt, serious pathology was not observed (Figures 4D and 4B, middle row). Finally, splenic T cells from OT-I transgenic *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice reconstituted with wild-type bone marrow exhibited markedly higher autoreactivity than controls as judged by proliferation of T cells in a "syngeneic" mixed lymphocyte reaction (Figure 4E).

Together, these experiments indicated that stromal cells of the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were sufficient to cause the T cell-mediated autoimmune condition observed in these mutant mice. Mutant hematopoietic cells, including T cells, do not appear to have intrinsic functional defects responsible for development of pathology. Rather, *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ stromal cells shape T cells to become mediators of the autoimmune condition. It is noteworthy that loss of NF-κB2 alone resulted in a mild tendency toward development of multiorgan inflammation, especially in the transfer models, although overt pathology was never observed.

Primary Defect in Thymic Stroma Is Responsible for Pathology in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice

Although stromal cells were shown to be critical for development of autoimmune pathology in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, it remained to be determined whether the defect was mediated by peripheral or by thymic stromal cells, or both. Because the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mutants were missing two genetic loci, it remained possible that two very different stromal cell types were affected. The thymic stroma is considered essential for negative selection of autoreactive T cells, resulting in part from the generation of tissue-restricted, peripheral self-antigens for presentation by these stromal cells or by crossprimed thymic dendritic cells (Anderson et al., 2005; Gallegos and Bevan, 2006). In contrast, autoreactive Treg cells are not eliminated and may even be positively selected by their recognition of self-antigens generated by and displayed on stromal cells (Aschenbrenner et al., 2007; Cabarrocas et al., 2006). To rigorously test whether the thymic stroma was sufficient to cause pathology in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, we carried out fetal thymic stroma transplants into athymic nude mice. Day 14.5 embryonic fetal thymi were collected from double knockout mice or NF-κB2-deficient control mice (including *Nfkb2*$^{-/-}$ *Bcl3*$^{+/-}$), and after a week in culture in the presence of 2-deoxyguanosine to eliminate all thymocytes, one or two thymic lobes were transplanted under the renal capsule of nude mice. (The outcome of the experiment was the same regardless of whether thymic lobes originated with *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ or *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I TCR transgenic animals. The latter mice were included as a source of thymic stroma to ensure that the outcome was not due to a potential transfer of undetectable numbers of surviving thymocytes. The transgene should prevent any T cells that might have been derived from the transferred thymocytes from causing pathology.) Between 5 and 8 weeks after transfer, 7 of the 9 nude mice engrafted with *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymus succumbed, whereas none of the 10 control transfers did (Figure 5A). The remaining two recipients of fetal thymi from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice succumbed later, whereas controls continued to survive, free of overt, serious pathology. Consistent with these results, nude mice with *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymi consistently developed substantial inflammatory infiltrates in lung and liver, whereas those with *Nfkb2*$^{-/-}$ thymi only sporadically developed mild infiltrates during this time frame (Figure 5B). Furthermore, splenic T cells in the nude recipients of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymi displayed a pronounced shift toward the effector and memory (CD44^{+}CD62L^{-}) phenotype when compared to control recipients receiving *Nfkb2*$^{-/-}$ thymi, even though these latter transplants already resulted in somewhat elevated numbers of the effector and memory cells when compared to normal wild-type mice (Figure 5C). Therefore, loss of both Bcl-3 and NF-κB2 in thymic stromal cells was sufficient to cause pathology, strongly suggesting that a single cell type was impaired by the combined loss.

Because NF-κB1 and NF-κB2 double deficiency resulted in complete lack of all lymph nodes, similar to that seen in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice (Franzoso et al., 1997b; Lo et al., 2006), we transferred fetal thymi from *Nfkb1*$^{-/-}$ *Nfkb2*$^{-/-}$ mice into nude mice. These nude recipients succumbed to inflammatory conditions by 5 to 8 weeks after transfer (not shown).

We also tested for autoantibody production in nude mice that had received thymi from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. The original donor *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice had a clear deficit in mature B cells (see above). Consistent with this deficit in mature B cells, we could not detect autoantibodies in the original donors (not shown). The (nonthymic) stromal and hematopoietic cells of nude recipients are genetically wild-type, so the presence of autoreactive T cells should stimulate autoantibody production by B cells. Initial analyses confirmed that nude mice with *Nfkb2*$^{-/-}$ *Bcl3*$^{-/-}$ thymi did indeed produce some autoantibodies (Figure 5D).

To investigate whether impaired T regulatory cell activity could have contributed to the inflammatory pathology, we cotransferred one WT (or *Nfkb2*$^{-/-}$) thymic lobe and one *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ lobe into nude mice and compared them to matched nude recipients that had received only one *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ lobe. The presence of the wild-type thymic lobe largely prevented organ infiltration (Figure 5E), and it reduced the relative number of CD44^{+}CD62L^{-} effector and memory cells in both the CD4 and CD8 compartments (Figure 5F). This result

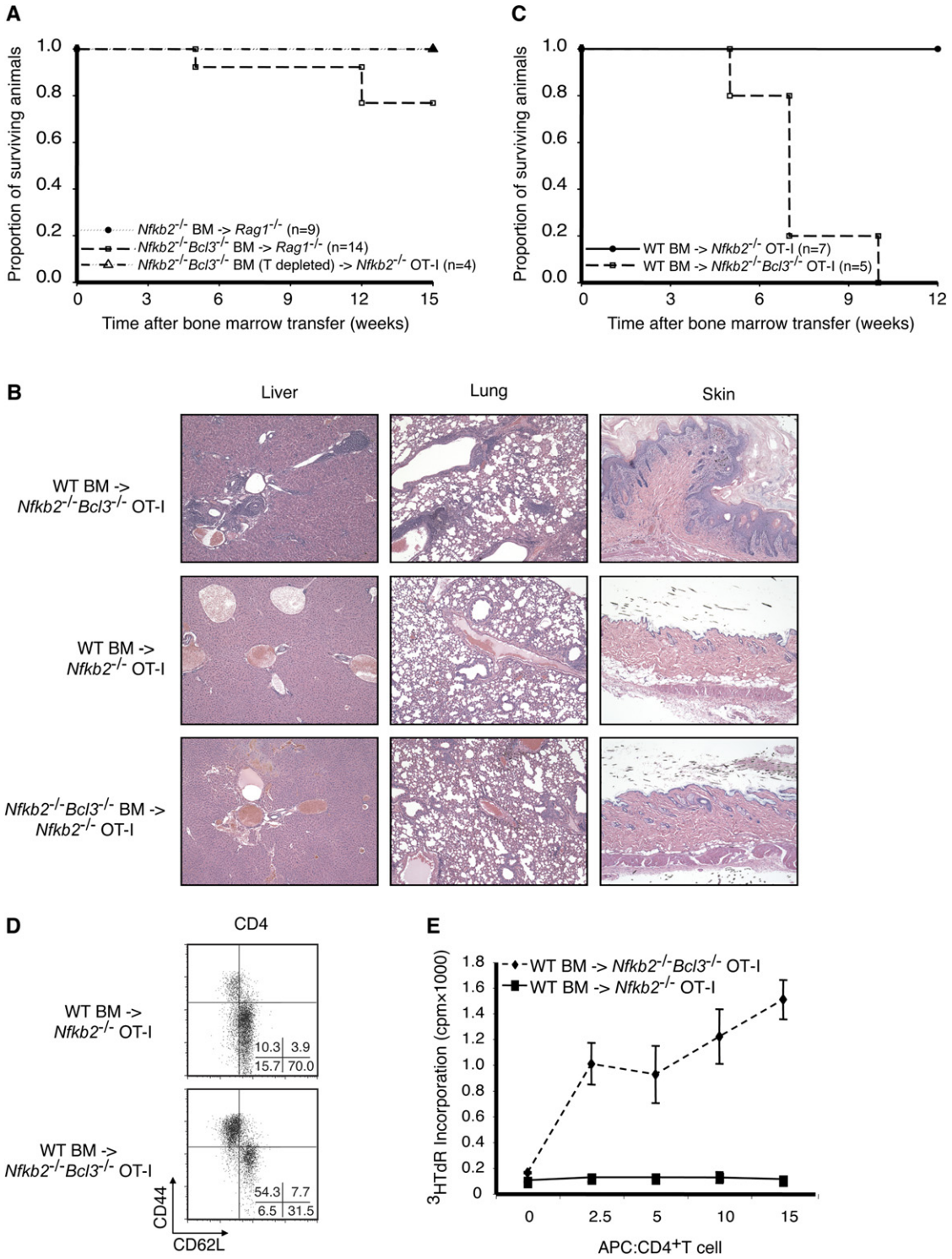

Figure 4. Stromal Cells Are Responsible for Fatal Multiorgan Inflammation and Increased Numbers of Effector and Memory T Cells in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice

(A) Bone-marrow (BM) cells from *Nfkb2*$^{-/-}$ and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were transferred into lethally irradiated *Rag1*$^{-/-}$ mice. In addition, *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ bone marrow was depleted of CD4^{+} and CD8^{+} T cells prior to transfer into *Nfkb2*$^{-/-}$ OT-I mice, as shown. Chimeric mice were monitored and the surviving proportion was plotted (see Figure 2B for further details).

(B) Liver, lung, and skin sections from BM chimeras were stained with H&E. Genotypes of BM donors and recipients were as indicated. Data in top two rows are representative of at least five independent experiments, bottom row representative of two independent experiments.

(C) Wild-type (WT) BM cells were transferred into lethally irradiated *Nfkb2*$^{-/-}$ OT-I and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I mice (analysis as in [A]).

suggests that the wild-type thymus generated a T regulatory activity that controlled the inflammatory potential of the T cells emerging from the mutant thymus. This further suggests the T regulatory activity of mutant thymi was in some way impaired because it was unable by itself to control organ infiltration.

Impaired Differentiation of Medullary Thymic Epithelial Cells

Having shown that the thymic stroma was sufficient to cause multiorgan inflammation, we investigated the thymic structures in the *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. The thymus contains medullary and cortical epithelium. Medullary thymic epithelial cells (mTECs) express Aire (autoimmune regulator), which has been implicated in expression of peripheral tissue-restricted autoantigens, such as preproinsulin-2 and salivary protein 1, and in presentation of such autoantigens (Anderson et al., 2005; Gallegos and Bevan, 2006). mTECs are thus important for negative selection of at least some autoreactive thymocytes. Thymic sections from 10-day-old mice were stained with the UEA-1 lectin, which detects a subset of mTECs (Surh et al., 1992), and with the DEC205 antibody, which preferentially stains cortical thymic epithelial cells (cTEC), while also weakly staining thymic dendritic cells (DCs) in the medulla (Figure 6A; Witmer-Pack et al., 1995). Additional sections were stained for other markers of medullary (MTS10, K14, ER-TR5, and G8.8; not shown) and cortical (ER-TR4) epithelium (Figure S2; Boyd et al., 1993; Farr et al., 1991; Godfrey et al., 1990; Klug et al., 1998; Van Vliet et al., 1985). The *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ sections appeared to lack fully differentiated medullary epithelial cells, because no clear staining was seen with UEA-1, ER-TR5, MTS10, or G8.8, whereas cTECs were readily detected with DEC205 and ER-TR4. There was, however, some weak staining with K14 in noncortical and thus presumably medullary regions in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ animals. Therefore, mTEC progenitor-like cells may have been present in medullary regions of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mutants, but these cells failed to fully differentiate. Of note, *Nfkb2*$^{-/-}$ thymi appeared to be partially impaired when compared to wild-type (or *Bcl3*$^{-/-}$) thymi, based on reduced staining with UEA-1, MTS10, and especially with G8.8 and ER-TR5. Partial impairment of the thymus in these mutant mice is consistent with mild inflammatory tendencies later in life and in transfer experiments (as shown here and Zhu et al., 2006).

In addition to a loss of differentiated mTECs and possibly as a consequence thereof, we also failed to detect thymic DCs in the medulla of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mutant thymic sections (Figure 6B, CD11c staining; the result was confirmed with MIDC-8 staining; not shown). The absence of thymic DCs may suggest a more generally impaired ability to negatively select T cells. Finally, natural killer T (iNKT) cell differentiation and presence in the periphery has been shown to depend on RelB expression in thymic stromal cells (Elewaut et al., 2003; Sivakumar et al., 2003). Consistent with this, we observed a severe loss of iNKT (CD1d-αGalCer tetramer^{+}) cells in liver and spleen of 2-week-old doubly deficient mice (data not shown).

To confirm that differentiated mTECs were not only phenotypically but also functionally deficient, we performed quantitative PCR analyses with RNA extracted from 10- to 12-day-old thymi for expression of several genes, including Aire, preproinsulin-2, salivary protein 1, and Foxn1 (Figure 6C). Thymus RNA from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice showed dramatically decreased amounts of Aire, insulin, and salivary protein 1, whereas expression of the control gene Foxn1 was essentially the same as in wild-type mice. Thymi from *Bcl3*$^{-/-}$ mice contained near wild-type amounts of mRNAs for Aire, preproinsulin-2, and salivary protein 1, whereas *Nfkb2*$^{-/-}$ mice expressed intermediate amounts of these mRNAs, suggesting a partial problem in the latter mutants, consistent with other evidence discussed above. These results show a profound loss of mTECs markers and function in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, presumably because of a block in differentiation of these cells, which in turn is likely to impair negative selection of autoreactive T cells that recognize self-antigens such as insulin and salivary protein 1. Loss of differentiated mTECs and thus expression of TSAs most likely also impacted Treg cells.

DISCUSSION

The data presented here implicate Bcl-3 and NF-κB in the control of immunological self-tolerance. Although Bcl-3 by itself is not absolutely necessary for central tolerance, its importance is revealed in the absence of NF-κB2. Mice lacking both NF-κB2 and Bcl-3, but not mice lacking one of these proteins, failed to survive past 4 weeks after birth, exhibiting severe multiorgan inflammation. Thus, NF-κB2 and Bcl-3 have functionally redundant roles in preventing the immune system from attacking its host. Interestingly, these proteins also have redundant functions during lymphoid organogenesis because only doubly deficient, but not singly deficient, mutants lack all lymph nodes.

Thymi of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ animals lack differentiated medullary thymic epithelial cells (mTECs) but do contain noncortical, medullary-like areas. Wild-type thymus contains clusters or networks of differentiated mTECs that can be identified with a limited set of surface markers, some of which differentiate subclasses of mTECs. mTECs are the source of various peripheral tissue-specific

(D) T cells from WT BM-reconstituted *Nfkb2*$^{-/-}$ OT-I and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I mice were gated on CD4^{+} and analyzed for expression of CD44 and CD62L by flow cytometry. Data representative of at least five independent experiments.
(E) T cells from WT BM-reconstituted *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ OT-I mice show elevated autoreactivity. APC, syngeneic antigen-presenting cells. The graph shows increasing ratios of APC to constant amounts of CD4^{+} T cells. Data are the mean of triplicates ± SD and similar results were obtained in two independent experiments; see Figure 2A for further details.
(B, D, and E) Tissues and cells were from mice 6–7 weeks after transfer.

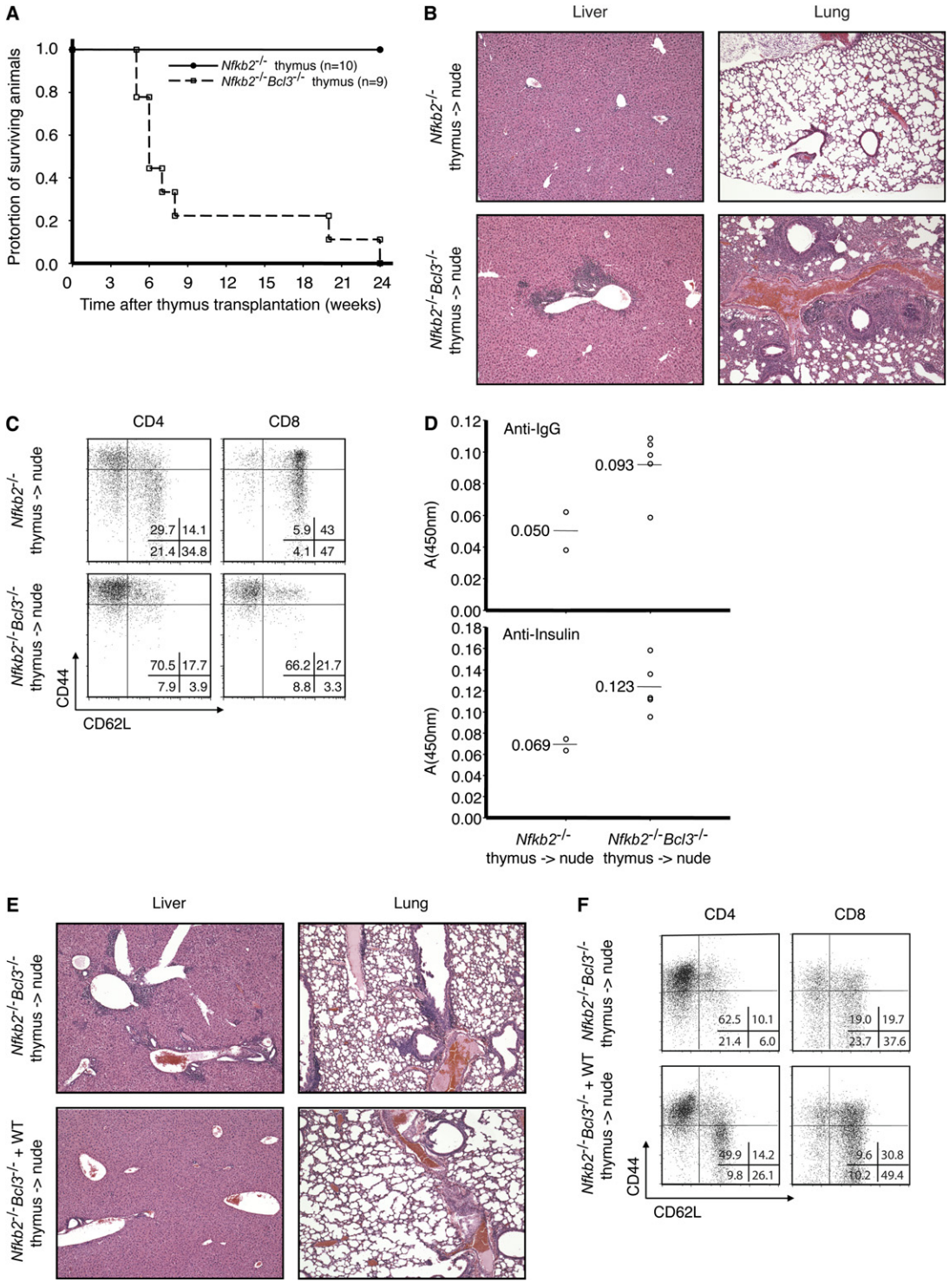

Figure 5. Engraftment of Athymic Nude Mice with Fetal Thymus Tissue from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice Causes Multiorgan Inflammation

(A) Nude mice engrafted with (T-depleted) fetal thymus lobes from *Nfkb2*$^{-/-}$ and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice (with and without OT-I transgene) were monitored and the surviving proportion was plotted (see Figure 2B for further details).

(B) H&E-stained sections from liver and lung of nude mice engrafted 6 weeks prior with fetal thymus from *Nfkb2*$^{-/-}$ or *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice.

(C) Splenic T cells from nude mice engrafted 5 weeks prior with fetal thymus from *Nfkb2*$^{-/-}$ or *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were gated on CD4^{+} and CD8^{+} and analyzed for expression of CD44 and CD62L by flow cytometry.

(B and C) Representative examples of at least five independent experiments are shown.

antigens (TSAs) in the thymus, such as insulin and salivary protein 1. mTECs may present these self-antigens directly to newly developing TCR$^+$ T cells or indirectly via cross-priming of local thymic DCs (Anderson et al., 2005; Aschenbrenner et al., 2007; Gallegos and Bevan, 2006). *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymi lack these differentiated mTEC clusters, as judged by the absence of surface markers and as judged also by lack of expression of insulin, salivary protein 1, and Aire. Expression of Aire is largely restricted to mTECs, where it functions as a transcriptional regulator for expression of TSAs. Aire may also have a more general role in presentation of self-antigens (Anderson et al., 2005). Lack of TSAs and Aire in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice is presumably a consequence of impaired differentiation of mTECs, although this does not rule out the possibility of a more direct role of NF-κB2 and Bcl-3. In addition to mTECs, *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice are missing thymic DCs. These cells normally reside in the medulla and the cortico-medullary junctions; they have been implicated in negative selection of T cells to self-antigens in general and to TSAs via crosspriming in particular (Aschenbrenner et al., 2007; Gallegos and Bevan, 2006; Gavanescu et al., 2007). Their absence is likely to be secondary to the absence of differentiated mTECs because we observed normal numbers of thymic DCs in lethally irradiated *Rag1*$^{-/-}$ mice whose hematopoietic compartment had been regenerated upon transfer of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ bone marrow (data not shown). We reason that lack of mTEC-dependent local expression of TSAs, coupled with lack of thymic DCs, in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice impairs negative selection of T cells recognizing TSAs and possibly also other antigens. Defective negative selection could be a primary cause of the observed fatal multiorgan inflammatory pathology.

The lack of differentiated mTEC clusters may also prevent the emergence of Treg cells that recognize TSAs. It has been postulated that Treg cells are positively selected in thymus by recognition of self-antigens presented by mTECs (Aschenbrenner et al., 2007; Cabarrocas et al., 2006). If so, our *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ animals may suffer from holes in the repertoire of their Treg cells for this class of self-antigens; such a defect may account for the observed reduction of thymic Treg cells shortly after birth. Treg cells emerging from the thymus have been referred to as natural Treg cells, differentiating them from adaptive Treg cells that may develop in the periphery in response to specific antigenic challenges (Bluestone and Abbas, 2003). In addition to possible holes in the natural Treg cell repertoire, it is conceivable that Treg cells were also functionally impaired in vivo at sites of inflammation, although nonspecific inhibitory activity did not appear to be compromised. The notion that Treg cell surveillance was somehow impaired in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice is supported by the finding that cotransplantation of wild-type thymus and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymus markedly reduced inflammatory pathology, presumably resulting from wild-type thymus-derived Treg cells exerting control over T cells emerging from the defective thymus. A deeper understanding of the development and functions of natural and adaptive Treg cells will be required to determine whether and how Treg cells of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice may have failed to control multiorgan infiltration.

Nfkb2$^{-/-}$*Bcl3*$^{-/-}$ mice bear striking similarities to mice lacking RelB and mice mutated in NIK (*aly/aly*). These mutant mice fail to develop lymph nodes, lack fully differentiated medullary epithelium, and develop multiorgan inflammation, although they differ in the severity of the pathology, with the present *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ exhibiting the most severe form (Kajiura et al., 2004; Weih et al., 1995). Defects intrinsic to various cell types have been suggested to contribute to the pathology of RelB-deficient mice (Martin et al., 2003; Xia et al., 1999). We found that transplantation of thymic tissue from RelB-deficient thymus caused multiorgan infiltration in nude mice (data not shown), similar to thymic tissue from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice, *aly/aly* mice, and IKKα-deficient mice (Kajiura et al., 2004; Kinoshita et al., 2006). Therefore, the severe inflammation observed in these mutant mouse models is due first and foremost to defects intrinsic to thymic stroma, and most likely to mTEC precursor cells, regardless of whether other cells were affected and contributed to the final phenotype as well.

The results obtained with mice lacking components of the nonclassical pathway (discussed above) clearly point out a crucial role of this pathway and its RelB target for induction of tolerance in the thymus. Activation of the nonclassical pathway in mTEC precursors must include signaling via the lymphotoxin β receptor (LTβR) because loss of this signaling route results in multiorgan inflammation as well (Boehm et al., 2003; Chin et al., 2003; Hehlgans and Pfeffer, 2005). It has recently been reported that the LTβR activates NF-κB only via the nonclassical, not the classical, pathway, so loss of NF-κB2 should abolish all LTβR signaling to NF-κB (Basak et al., 2007). Nevertheless, *Nfkb2*$^{-/-}$ mice have a much milder phenotype than might be predicted from this, with only mild sporadic inflammation after at least 6 months of age, as described here and elsewhere (Zhu et al., 2006). The likely explanation is that RelB complexes can still be activated in *Nfkb2*$^{-/-}$ mice, because only these mutants lack the p100 inhibitor. We have shown previously that TNFα strongly activates p50-RelB in *Nfkb2*$^{-/-}$, but not wild-type cells, and that it does so via classical pathway-induced expression of both RelB and NF-κB1 (Muller

(D) Nude mice engrafted with fetal thymus from *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice exhibited elevated autoantibodies when compared to nudes engrafted with *Nfkb2*$^{-/-}$ fetal thymus. Serum was collected from matched nudes between 5 and 7 weeks after transplantation. Serum antibody titers to the indicated autoantigens were determined with ELISA assays (1:100 dilution for anti-IgG, 1:200 dilution for anti-insulin; p = 0.047 and p = 0.03, respectively).
(E) H&E-stained sections from liver and lung of nude mouse engrafted 10 weeks prior with one fetal thymic lobe from a *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mouse or with the other fetal thymic lobe from the same *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mouse and one lobe from a WT mouse.
(F) Splenic T cells were isolated from the thymic stroma-engrafted nude mouse shown in (E) were gated on CD4$^+$ and CD8$^+$ and analyzed for expression of CD44 and CD62L by flow cytometry.
(E and F) The engrafted nude mouse is a representative example of six other such matched transfers.

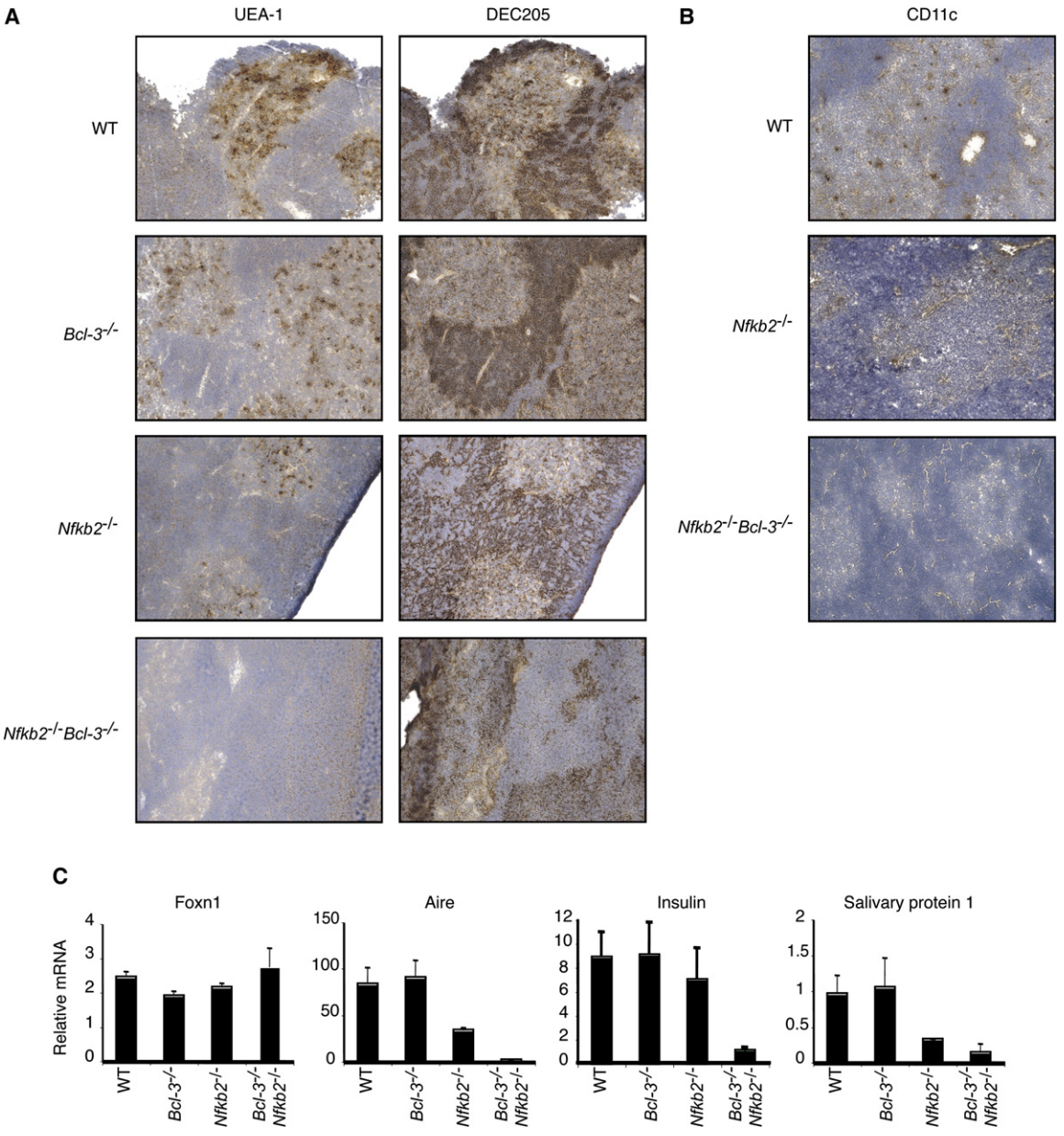

Figure 6. Disrupted Thymic Architecture and Phenotypic and Functional Loss of Medullary Thymic Epithelial Cells in *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ Mice

(A) Thymic tissue sections of 10-day-old wild-type (WT), *Bcl3*$^{-/-}$, *Nfkb2*$^{-/-}$, and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were stained with UEA-1 (mTECs) and the DEC205 antibody (primarily staining cortical TECs, but also thymic dendritic cells).

(B) Thymic tissue sections of 14-day-old WT, *Nfkb2*$^{-/-}$, and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were stained with CD11c (thymic dendritic cells).

(A and B) Representative examples of at least four independent experiments are shown.

(C) Quantitative PCR analysis of RNA from 10- to 12-day-old WT, *Bcl3*$^{-/-}$, *Nfkb2*$^{-/-}$, and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ thymi for expression of Foxn1 (control), Aire, and the peripheral tissue antigens salivary protein 1 and preproinsulin-2 (Insulin 2). Data are shown as the mean ± SD of 3–5 mice per group.

and Siebenlist, 2003). We therefore wondered whether Bcl-3 might be required for TNFα-induced activation of p50-RelB in *Nfkb2*$^{-/-}$ cells, which could explain the dramatic phenotype of *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice. However, we were unable to detect any role for Bcl-3 in TNFα-induced p50-RelB activity (data not shown). We conclude that in the present biologic context, Bcl-3 likely functions by directly regulating target genes, not indirectly via global effects on NF-κB activity. The genes involved in the differentiation of mTECs are not known. Future work will have to identify such genes, those among them targeted by Bcl-3, RelB, or any of the NF-κB factors, and the molecular mechanisms by which these factors regulate their expression.

To date, little is known about physiologic targets of Bcl-3 in any biologic context; furthermore, actual mechanisms of recruitment to and functions within chromatin of potential direct targets remain unknown or unconfirmed, although Bcl-3 does strongly interact with homodimers of p52 (NF-κB2) and p50 (NF-κB1). In the present biologic context, Bcl-3 must function independent of p52, but could still function in concert with p50. There is indirect support for a model wherein Bcl-3's contributions to tolerance might be mediated via p50, as indicated by the fact that RelB, NF-κB1 doubly deficient mice develop a more severe form of multiorgan inflammation than mice deficient in RelB only (Weih et al., 1997). Therefore, Bcl-3 and p50 may interact to help differentiate mTECs and induce tolerance.

It also remains to be determined how and by which signal Bcl-3 is activated during mTEC differentiation. In addition to LTβR signals, differentiation of mTECs must involve at least one other signal. Thymi of TRAF6-deficient mice lack differentiated mTECs, and their transplantation into nude mice induces multiorgan inflammation, even though TRAF6 is not a known component of the LTβR pathway (Akiyama et al., 2005). The TRAF6-mediated signal may, however, contribute indirectly to the nonclassical pathway by inducing expression of RelB. Bcl-3 and RelB could both lie downstream of the TRAF6-associated signal. TRAF6 can activate the classical NF-κB pathway, which is known to induce expression of Bcl-3 (Ge et al., 2003).

The present work suggests extensive parallels between thymic stromal cells, specifically mTECs, and stromal cells of secondary lymphoid organs, specifically follicular dendritic cells (FDCs). FDCs and mTECs are highly differentiated cells that serve as APCs for B and T cells, respectively. Based on the above data, Bcl-3 and NF-κB2 are critically involved in the differentiation of both cell types, as are the nonclassical pathway and RelB. In addition, differentiation of mTECs and FDCs is partially controlled by LTβR ligands expressed on interacting T or B lymphocytes, respectively (Boehm et al., 2003; Chin et al., 2003).

The results presented here reveal an unanticipated role of Bcl-3 in central tolerance, which emerges in the absence of NF-κB2. Bcl-3 functions redundantly with NF-κB2; it partially compensates for loss of NF-κB2 and thus loss of the nonclassical pathway of activation. Based on these data, it will be important to investigate whether Bcl-3 is deregulated in human autoimmune diseases. It will also be of interest to explore possible connections between Bcl-3's roles in tolerance induction and tumorigenesis. Bcl-3 has been implicated as an oncogene in a variety of hematologic and solid tumor malignancies (Kashatus et al., 2006; Massoumi et al., 2006). Further work is needed to elucidate the precise molecular mechanisms and targets of Bcl-3 underlying its various roles.

EXPERIMENTAL PROCEDURES

Mice

Bcl3$^{-/-}$ and *Nfkb2*$^{-/-}$ mice were described previously (Franzoso et al., 1997a, 1998). Both have been backcrossed to C57 BL/6 for 11 or more generations. *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice were obtained by crossbreeding single-deficient mice. Mice were housed in NIAID Institute facilities, and all experiments were done with approval of the NIAID Animal Care and Use Committee and in accordance with all relevant institutional guidelines.

Antibodies and Flow Cytometry

Flow cytometric analyses were performed on freshly prepared, erythrocyte-lysed single-cell suspensions of thymocytes or splenocytes in PBS/0.5% BSA/2 mM EDTA. Stained cells were analyzed on a FACSCaliburTM (BD Biosciences) with the following: FITC-, PE-, PerCP-, or allophycocyanin-conjugated monoclonal antibodies purchased from BD Biosciences (clone name in parentheses). For surface marker staining, single-cell suspensions from thymus and spleen were preincubated with anti-FcγIII/II receptor (2.4G2) to block Fc receptors, and then stained with anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14). For Foxp3 intracellular staining, cells were fixed after surface staining and then stained with anti-Foxp3 (FJK-16 s) (eBioscience). Data were analyzed with FlowJo software (Tree Star Inc.).

Cell Purification

Erythrocyte-lysed, single-cell suspensions from spleen served as splenocytes. For autoreactive assays, $CD4^+$ T cells were purified from splenocytes by negative selection on midi MACS (magnetic cell separation) columns (Miltenyi Biotec) according to the manufacturer's instructions. Antigen-presenting cells (APCs) were prepared from wild-type splenocytes by depletion of T cells with anti-CD4 and anti-CD8 beads with MACS. For isolation of $CD4^+CD25^+$ T cells or $CD4^+C25^-$ T cells, $CD4^+$ T cells obtained by negative selection with MACS (see above) were incubated with Fc receptor-blocking antibody 2.4G2, then stained with FITC-anti-CD4, PE-anti-CD8, and PerCP-anti-CD25, and finally sorted with a FACSAria cell sorter (BD Biosciences).

Adoptive Transfer of $CD4^+$ T Cells

5×10^6 $CD4^+$ T cells, purified from splenocytes by negative selection (see above), were injected intravenously into sublethally irradiated (500 rads) male *rag1*$^{-/-}$ mice. Mice were weighed weekly and monitored daily.

Bone-Marrow Chimeras

Bone-marrow cells were harvested by flushing mouse femurs and tibias with cold Hank's Balance Salt Solution (HBSS). Bone-marrow cell suspensions were depleted of T cells by magnetic cell separation with beads conjugated to anti-CD4 and anti-CD8 (Miltenyi Biotec). $3–5 \times 10^6$ erythrocyte-lysed bone-marrow cells were injected into the tail veins of lethally irradiated (900 rads) recipient mice in 0.2 ml HBSS. Mice were weighed weekly and monitored daily.

T Cell Assays

For mixed lymphocyte reactions, 1×10^5 negatively selected $CD4^+$ T cells per well were incubated with 5×10^5 (unless otherwise specified) syngeneic, T cell-depleted, irradiated splenocytes (3000 rads) serving as (self-) antigen-presenting cells (APCs) in 96-well plates for 72 hr and pulsed with 1 μCi of [^{3}H]thymidine per well for final 8 hr. T regulatory cell-mediated suppression assays were performed under the same conditions, except with 5×10^4 $CD4^+CD25^-$ T cells as responders, 2.5×10^5 APCs, anti-CD3 (1 μg/ml) (BD Biosciences), together with 2-fold dilutions of $CD4^+CD25^+$ T regulatory cells (as specified).

Autoantibodies

For titers of antibodies to IgG and insulin (Sigma), 96-well plates were coated with these antigens (10 μg/ml in phosphate-buffered saline) at 37°C for 2 hr. Plates were subsequently washed 3 times and then incubated with blocking buffer (BBS, 0.05% Tween 20, 1 mM EDTA, 0.25% NaN_3) at room temperature for 30 min. After three rinses, the plates were incubated with 50 μl diluted sera (1:50, 1:100, 1:200) then incubated at room temperature for 2 hr. Antibodies were detected

with HorseRadish Peroxidate (HRP)-conjungated rat anti-mouse IgG or IgM (Jackson Immunoresearch).

Histopathology and Immunohistochemistry

Organs and tissues were immersion fixed in 10% buffered formalin and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Thymi were embedded in OCT freezing solution. Cryostat sections were fixed for 10 min in ice-cold acetone, then sequentially blocked with peroxidase blocking reagent (DakoCytomation), avidin/biotin blocking kit (Vector Laboratories), and blocking buffer containing 10% rabbit or goat serum and incubated with biotinylated ulex europaeus agglutinin 1 (UEA-1; Vector Laboratories) or antibody to DEC205 (Serotec), MTS-10 (gift of R. Boyd), Keratin 14 (Covance), ER-TR4 and ER-TR5 (gift of W. van Ewijk), MIDC-8 (Covance), and CD11c (BD Biosciences) at 4°C overnight. Staining was developed directly or after incubation with the appropriate biotinylated second step antibody via StreptAB-Complex-HRP (streptavidin complexed with biotinylated peroxidase), according to the manufacturer's recommendations (Vector Laboratories).

Fetal Thymus Transplantation

Thymic lobes were isolated from timed embryos 14.5 days after coitus and were cultured for 6–7 days on Nucleopore filters placed in complete medium containing 1.35 mM 2-DG (Sigma), then washed twice for 2 hr each. Thymic lobes were transplanted under the renal capsule of adult (6–8 weeks of age) male B6/nu mice.

RNA Isolation and Real-Time PCR

Thymi were frozen and ground on dry ice, and powder tissue was used for RNA isolation. 1 ml of TRIzol (Invitrogen) was added to 100 mg of powder tissue and homogenized with a needle syringe, and then 200 μl of chloroform was added and mixed by vortex. Aqueous phase was harvested to a new tube after 15 min centrifugation at high speed, and then 500 μl of isopropanol was added. RNA preparation was incubated 10–20 min on ice and followed by 10 min centrifugation at high speed. RNA Pellet was dissolved in 100 μl of DEPC-H2O and the RNA cleanup/Dnase digestion protocol of QIAGENe was followed. 2 μg RNA was used for cDNA synthesis with superscriptII RT (Invitrogen).

For quantification of gene expression with real-time PCR, 1 μl of the cDNA reaction mix was used with the platinum quantitative PCR super Mix-UDG and LUX primers (Invitrogen), according to manufacturer's instructions for the Corbett Rotor-Gene real-time machine.

LUX primers were designed with D-LUX Designer software available on the Invitrogen Web site. The primer sequences were as follows. GAPDH: 5′-CACCATCGTCCCGTAGACAAAATGG[FAM]G-3′, 5′-CAAATGGCAGCCCTGGTGA-3′; Foxn-1: 5′-CCACTCTTCCCAAAGCCCATC-3′, 5′-CGGAACACTGACTGGAAGGCTTC[FAM]G-3′; Aire: 5′-CGTGACGGACGACTCTGCTAGTCA[FAM]G-3′, 5′-GCAGGATGCCGTCAAATGAGT-3′; Salivary protein-1: 5′-CAACACTCCTGGCACTCCTTGTG[FAM]TG-3′, 5′-CTGTTTGTCTCCGGGTCCTG-3′.

For quantification of preproinsulin 2, 1 μl of cDNA reaction synthesis was used with platinum SYBR green quantitative PCR super Mix-UDG (Invitrogen) and the following primers: Preproinsulin2: 5′-GCCCTAAGTGATCCGCTACAATC-3′, 5′-TCTACAATGCCACGCTTCTGC-3′; β-actin: 5′-GTGGGCCGCTCTAGGCACCAA-3′, 5′-CTCTTTGATGTCACGCACGATTTC-3′.

Supplemental Data

Two figures are available at http://www.immunity.com/cgi/content/full/27/3/438/DC1/.

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